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The overall goal of this program was to characterize the serum factor(s) which regulated steroid hormone dependent human breast cancer cell growth. This included an identification of the molecule(s) and studies to establish the endocrine physiology of the regulator(s) as well as partial characterization of the receptors mediating serum factor action. We have discovered that two plasma glycoproteins, SHBG Type II and CBG, are negative regulators (*i.e.* inhibitors) of estrogen receptor positive (ER^+) and progesterone receptor positive (PR^+) breast cancer cell growth, respectively, and that autonomous (ER^- and PR^-) breast cancer cells are not inhibited by these glycoproteins. The new form of sex hormone binding globulin has been designated SHBG Type II. It has not previously been identified or characterized from any source. The discovery of a new regulatory role for CBG in breast cancer growth may provide our first molecular link between increased stress and an elevated risk of breast cancer.

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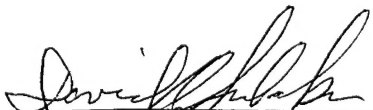

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I. TABLE OF CONTENTS

5. INTRODUCTION

A. Literature Background

1. Growth Factor Related Background

This project is based on recent discoveries in our laboratory which have resolved a longstanding problem in breast cancer endocrine physiology. In 1896, Beatson reported that oophorectomy had palliative effects on breast cancer (1). That report first implicated estrogens and progestins in breast cancer growth. Since that time, researchers have identified specific nuclear receptors for these steroid hormones (2-3) and characterized them by cDNA cloning and many other methods (4). This advance led to the assumption that the interaction of estrogens and progestins with their specific nuclear receptors was sufficient to explain target tissue growth (2-3). However, as work progressed, this point of view became less defensible.

Many investigators came to the conclusion that other regulatory molecules also were required. For example, our laboratory proposed that plasma contained an estrogen regulated mediating "estromedin" which controlled target cell proliferation (5). Several putative estromedins were purified from tissues and identified as insulin-like growth factors I and II (IGF-I and IGF-II) (6,7), transforming growth factor α (TGFα) (8), and fibroblast growth factors (9). None proved to be related to estrogen dependent breast cancer cell growth (10). Another laboratory proposed that plasma contained an inhibitor (estrocolyone) which negatively controlled target tissue growth (11). It has been proposed that estrocolyone inhibits growth by binding to target cells, and that this restraint is reversed when steroid hormones associate with the inhibitor to release it from cells. Estrocolyones have proven to be unstable during isolation and because of this have not been characterized at the molecular level (12). Another group concluded that serum albumin was "estrocolyone" (13). This hypothesis was not confirmed (10). Continuing with the theme of serum factor regulation, other investigators did not offer specific names for the agents involved, but did conclude that serum contained a critically important regulator(s) which had not yet been identified (14-17). In support of this viewpoint, it was known that estrogen mitogenic effects *in vitro* were difficult to demonstrate (17-20), especially in completely serum-free hormonally defined medium (10,21,22). When estrogen mitogenic effects were found *in vitro*, they were most pronounced in serum supplemented cultures (11,17,23-26). Recently, we found that the ferric iron in culture medium was deleterious to hormone-responsive growth (27). Use of "low-iron" medium now permits identification of very large estrogen mitogenic effects in serum-supplemented culture (27).

The work of Sporn and Todaro (28) opened new possibilities with regard to growth factors and cancer. They proposed that growth factors might act as autocrine, paracrine or endocrine regulators. Many groups joined in these studies. For example, one consortium of investigators proposed that estrogens induced TGFα , IGF-I and/or IGF-II, and that these acted as autocrine, paracrine or intracrine agents to promote breast cancer growth (29-35). Despite the attractiveness of these hypotheses, such mechanisms have yet to be established conclusively. Data from the original laboratories and others (36-39), and from our group (10), have raised serious questions concerning participation of these growth factors in estrogen-dependent growth.

2. SHBG Type I and CBG Related Background

To understand what we have achieved, it is necessary to point out that SHBG has been known for many years. It was first purified from human plasma in 1975 by androgen affinity chromatography. SHBG is a M_r 94,000 homodimer (40,41). SHBG is the major carrier of sex hormones in plasma (42-45). For the purposes of this discussion, we have named this well established form **SHBG Type I**. Likewise, CBG has been well characterized. It is a M_r 58,000 monomer which transports progestins and corticosteroids in plasma (42).

Initially, most investigators thought that SHBG Type I and CBG had no functions other than to serve as inert transport proteins which regulated the relative levels of "free/active" versus "bound/inactive" steroid hormones in plasma (42,46). This notion prevailed until recently when investigators began to investigate the interactions of these carriers with target tissues. Evidence began mounting that these proteins might have direct cellular functions. High affinity specific receptors for SHBG Type I were found on sex hormone target tissue cells (47-57). Also, receptors for CBG were identified on human breast cancer cells and other target tissues (42,58,59,61). Even with these observations, the function of these sites was a mystery.

To resolve this issue, several groups asked if these receptors might be docking sites for delivery of steroid hormones into the interior of cells. Currently, the majority of evidence suggests this is unlikely (42,49). Instead, these same investigators found that the receptors for both SHBG Type I and CBG appeared to have transmembrane signaling functions which implied regulatory roles. It was found that only the steroid hormone free forms of the carriers bind to membrane receptors. If the transport proteins already have steroid hormone bound, they did not associate with their receptors. However, if the steroid hormone free forms of either SHBG Type I or CBG were already associated with their respective receptors, they could also accept an appropriate steroid hormone. When this happened, there resulted a transient increase in intracellular cyclic AMP (42,55,59,60). While these observations have not been linked to growth, they cannot be ignored as evidence that SHBG Type I and CBG may have functions beyond being inert plasma transport proteins.

Most of what is known about SHBG Type I and CBG receptors has come from the study of ^{125}I-SHBG Type I or ^{125}I-CBG specific binding to whole cells or detergent solubilized membranes. The SHBG Type I receptor from prostate cells showed a ^{125}I-SHBG Type I affinity of K_a ~3 x 10^{10} M^{-1} (54,55). Studies with ^{125}I-CBG and human breast cancer MCF-7 cells gave K_a of ~ 1.4 x 10^9 M^{-1} (42,59). An M_r 170,000 for the SHBG Type I and CBG receptors has been reported (52,54). Another study showed that amino acid residues 48→57 of SHBG Type I contained a receptor recognition sequence (56). **Despite these analyses, no structural information (*e.g.* cDNA sequences) is available for the CBG receptor or the receptor(s) for any type of SHBG.**

B. Scope/ Purpose and Perspective

During the first 24 months of this project, we have addressed one of the major unresolved issues in breast cancer endocrine physiology. We proposed in our original

application to identify the serum factor(s) which regulated estrogen-dependent breast cancer cell growth. We have accomplished this goal.

The work supported by US Army grant DAMD17-94-J-4473 has provided a new perspective on the issue of what molecules regulate steroid hormone dependent growth. We have discovered that two plasma glycoproteins, SHBG Type II and CBG, are negative regulators (*i.e.* inhibitors) of estrogen receptor positive (ER^+) and progesterone receptor positive (PR^+) breast cancer cell growth, respectively, and that autonomous (ER and PR) breast cancer cells are not inhibited by these glycoproteins. The new form of sex hormone binding globulin has been designated SHBG Type II. It has not previously been identified or characterized from any source. The discovery of a new regulatory role for CBG in breast cancer growth may provide our first molecular link between increased stress and an elevated risk of breast cancer.

To place our study in perspective, it is vital to recall that the loss of negative regulation already has been implicated in a striking increase in susceptibility to breast cancer. For example, the autosomal dominant BRCA1 gene appears to code for a suppressor protein (62-64). Mutations in BRCA1 confer a risk of 85% to breast and ovarian cancer. This autosomal dominant trait is carried by about 5% of the women in the United States. The BRCA2 gene likewise may have a role in breast cancer development although it is open to question how important this locus is beyond a small group of women. Also, the loss of function (*i.e.* mutations) of the p53 tumor suppressor protein has important implications for germline and "sporadic" (*i.e.* non-familial) breast cancer susceptibility (65-68). It is notable that sporadic breast cancers represent the large majority of new diagnoses of this disease. As yet, we have no clear understanding of the mutations involved in these cancers.

Our study suggests new loci for evaluation of susceptibility to"sporadic" breast cancer. It is possible that mutations, or even some natural variations, in the structures of SHBG Type II or CBG may predispose women to breast cancer. Changes in primary structure which reduce receptor affinity can be expected to alter negative control. Indeed, it is well established that rapidly growing tissues are highly susceptible to carcinogenesis. Our findings suggest a reevaluation of the thought that only intracellular signaling changes/mutations are important in breast cancer. Our project offers the possibility of exploring alterations in extracellular negative regulatory signals as critically important sites for genetic and physiological changes leading to an increased risk of breast cancer.

6. BODY OF PROGRESS REPORT

A. Original Statement of Work

(See the next page)

PLEASE NOTE: ***The TASKS emphasized by italics print have been completed or are in progress as of September 14, 1996.***

PI: David A. Sirbasku, Ph.D.

TASK 1. Purification of Estrogen Mediating Serum Factor (EMSF): Months 0-30

(a) Purification will be done by application of conventional, FPLC and HPLC methods using horse and, if indicated, human serum.
(b) N^{α} and internal amino acid microsequencing will be done.
(c) Computer registered sequences will be used to identify EMSF.
(d) Rabbit polyclonal antisera will be raised against ovalbumin conjugates of EMSF peptides synthesized by solid phase methods.
(e) Immunoabsorption will be used to confirm the relationship between amino acid sequence and activity.
(f) The tissue of origin of EMSF will be sought in mouse organ extracts by Northern blotting for m-RNA.
(g) The effects of estrogen status on EMSF concentration in mouse blood will be assessed by ELISA or RIA.
(h) cDNA libraries will be screened for EMSF containing plasmids.
(i) If the complete sequence of EMSF is not known, we will determine it by dsDNA dideoxy chain termination sequencing.

TASK 2. Characterization of the EMSF Receptor: Months 12-48

(a) ^{125}I-EMSF will be made by the chloramine T reaction.
(b) Specific ^{125}I-EMSF binding will be investigated with estrogen sensitive breast cancer cells. The experimental parameters will be time, concentration, temperature, and effects of estrogens in the culture medium. Scatchard analysis is expected to give K_D values and numbers of sites per cell.
(c) Chemical cross-linking with ^{125}I-EMSF followed by SDS-PAGE and autoradiography will be used to identify the M_r of the specific ligand binding receptor.
(d) Internalization will be studied to ask if EMSF undergoes receptor mediated endocytosis.
(e) The same studies outlined in (a) will be done to determine if estrogen insensitive breast cancer cells lack EMSF specific binding.
(f) MAbs will be raised which block ^{125}I-EMSF binding to receptors.
(g) The Mabs will be used to screen cDNA libraries from estrogen sensitive cells to obtain plasmids for dideoxy dsDNA sequencing of the complete receptor.
(h) Transfection with full length receptor cDNA will be evaluated to determine if estrogen insensitive cells can be reverted to negative control by EMSF.

TASK 3. Physiological Significance <u>in vitro</u> and <u>in vivo</u>: Months 30-48

(a) The MAbs raised will be evaluated as growth agonists and antagonists in serum-free culture with estrogen sensitive breast cancer cells.
(b) The effects of antagonistic MAbs will be investigated on estrogen sensitive and insensitive breast cancer cell tumor formation athymic nude mice.

B. Review of Progress Based on Statement of Work

1. Task 1(a) through 1(c): *(a) Purification will be done by application of conventional, FPLC and HPLC methods using horse and, if indicated, human serum. (b) N^{α} and internal amino acid microsequencing will be done. (c) Computer registered sequences will be used to identify EMSF.*

Before undertaking this task, we needed a fully developed growth bioassay for the activity we were seeking. This meant using a cell line which was fully characterized and positively estrogen dependent in culture. Our MTW9/PL2 rat mammary tumor cell line fit these requirements.

The MTW9/PL cell line was established in culture from the carcinogen-induced hormone-responsive MT-W9A rat mammary tumor and shown to form estrogen, androgen and progesterone responsive tumors in Wistar-Furth rats. It was used to derive the MTW9/PL2 cells which also exhibit estrogen-responsiveness *in vivo*. In this study, we describe serum supplemented cell culture conditions in which the MTW9/PL2 cell line demonstrates steroid hormone growth responsiveness. The serum used in the cell cultures was steroid hormone-depleted by charcoal-dextran treatment at 34°C. When increasing concentrations of hormone-depleted serum were added to MTW9/PL2 cell cultures, growth was inhibited progressively. Between 30 and 50% (v/v) hormone-depleted serum suppressed growth completely. Addition of 1.0×10^{-13} M to 1.0×10^{-8} M 17β-estradiol (E_2) reversed the serum dependent inhibition. Under optimal conditions, estrogen induced growth was 2^5 to 2^6 or 32 to 64-fold greater than in control cultures. Even when the cells had been inhibited for six days, E_2 addition restored growth. At 1.0×10^{-11} to 1.0×10^{-7} M, estrone, estriol and the synthetic estrogen diethylstilbestrol promoted optimal growth. Testosterone and dihydrotestosterone were nearly as effective but only at $\geq 10^{-7}$ M. Progesterone was partially effective at $\geq 1.0 \times 10^{-6}$ M. Cortisol was only marginally effective or not effective. By labeled hormone analysis, MTW9/PL2 cells have high affinity receptors for estrogens and progesterone but not androgens or glucocorticoids. Western immunoblotting identified estrogen and progesterone receptors but not androgen receptors. The serum from the adults of several species including rat and human demonstrated activity. Fetal bovine and fetal equine serum showed little activity. The studies described show that the same pattern of hormone-responsiveness seen with MTW9/PL cells in rats is now demonstrable in culture. Furthermore, the MTW9/PL2 cells in culture are the most steroid hormone growth responsive of any of the rat mammary tumor lines yet reported.

Because this cell line so very closely mimics those of human breast, it was used for all of our initial studies of the serum factor identity as SHBG and CBG. Key results were checked with the estrogen and progesterone responsive T47D human breast cancer cell line. This approach saved about six moths during the first year. The rat MTW9/PL2 bioassays require 7 days. The T47D assays required 14 days.

We set out to purify this serum factor. We chose horse serum because of its high specific activity and easy availability at low cost. Also it was not as serious a biohazard as human serum.

The purification of the serum factor mediating the steroid hormone effects on responsive cancer cells initially was a very perplexing problem. The factor(s) was completely stable in whole serum for three weeks at 4°C, and yet remarkably unstable during purification. After a few months

of frustration, we found that simple dialysis caused inactivation. Ultrafiltration proved that the factor was not lost in the dialysate, and therefore, must be a high molecular weight entity. Further exploration revealed that the "factor" had a M_r of about 180,000. It was acid and urea labile and lost when heated at 60°C. Chelex treatment caused inactivation, which suggested metal ion stabilization. A study of metal ions revealed that calcium at 0.1 to 50 mM stabilized the activity. Other metals were not effective. From these constellation of properties, we thought that the activity in serum might be SHBG. Western immunoblotting with anti-human SHBG confirmed that the activity and SHBG coincided in the same fractions from molecular sieve chromatography.

However, two major facts contraindicated SHBG. First, we were using horse serum for our studies. It was not thought to have SHBG. Also, we were assaying with rat mammary tumor cells. Adult rats were not thought to have plasma SHBG. Therefore, we concluded that the calcium stabilized factor(s) might be related to SHBG immunologically, but it was not functionally equivalent to the well known form of SHBG.

We set out to purify the activity from horse serum. What we found were two activities, both of which were physiologically highly relevant. Corticosteroid-binding globulin (CBG) and a new form of sex hormone-binding globulin (SHBG) were purified from horse serum by sequential cortisol-agarose affinity and Phenyl-Sepharose chromatography. The preparations were ≥ 90% homogeneous as estimated by sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Coomassie blue staining. Using reducing SDS-PAGE, an M_r 60,000 was estimated for CBG. Sucrose gradient sedimentation under non-denaturing conditions identified two CBG forms of M_r 43,000 and 139,00. Tritium labeled steroid hormone binding to CBG was investigated at 34°C. Cortisol, progesterone, dihydrotestosterone (DHT) and 17β-estradiol (E_2) showed decreasing association constants (K_a) of 1.41×10^8, 2.13×10^7, 1.64×10^5 and 9.51×10^4 M^{-1}, respectively. Amino acid sequencing of five trypsin generated peptides of CBG showed 79% homology with human, rabbit or rat CBG as determined by computer assisted analysis. These studies affirmed that horse CBG was very similar to CBG from other species. In contrast, horse SHBG was distinctly different than SHBG from other species. SDS-PAGE analysis of horse SHBG identified subunits of M_r 60,000, 58,000 and 29,000. Western analysis done with an antibody against human SHBG showed cross-reaction with all three subunits. Sucrose gradient sedimentation of horse SHBG under non-reducing conditions revealed a single M_r 108,000 form that included all three subunits. Chemical cross linking followed by sucrose gradient sedimentation and SDS-PAGE confirmed that all three subunits were part of the M_r 108,000 complex. The binding of labeled DHT, E_2, cortisol and progesterone at 34°C to horse SHBG gave decreasing association constants (K_a) of 6.97×10^7, 3.55×10^7, 5.30×10^5 and 1.27×10^4 M^{-1}, respectively. In contrast to SHBG from other species, horse SHBG was N^{α} blocked. Amino acid sequencing of fragments of horse SHBG generated by chemical and enzymatic treatment showed that ten peptides (94 residues) averaged only 33% homology to human or rabbit SHBG or to the related to rat androgen binding protein as determined by computer assisted analysis. Based on the results presented, we have concluded that the properties of horse SHBG are significantly different than those of SHBG from other species. For these reasons, we have tentatively designated the horse serum protein as SHBG Type II.

Routinely, we are able to isolate 5 to 8 mg of SHBG Type II and 26 to 30 mg of CBG from two liters of 34°C charcoal-dextran extracted horse serum. By our present conventional

method, this process requires three weeks. During this year, the US Army Breast Cancer Program approved a supplemental request of $36,058 to purchase a fully automated FPLC. We are now in the process of getting the equipment operating. We anticipate being able to complete one preparation in eight days or less with the new FPLC. This will have a very beneficial effect on our research since larger supplies of both proteins are needed to conduct the serum-free culture work and to carry out the receptor characterization studies.

Next, we addressed the issue of whether rats have SHBG. Adult rats are known to have serum corticosteroid binding globulin (CBG) but are thought not to have SHBG. However, studies in our laboratory suggested that a relatively high affinity sex hormone binding sites were identifiable in the serum of adult males and females. To further characterize these binding sites, we set out to isolate this SHBG-like protein using a modification of the purification protocol that had been used above to isolate horse SHBG Type II. Pooled adult male and female rat serum was submitted to the two step procedure with was sequential Cortisol-Agarose and Phenyl-Sepharose chromatography. This method yielded 2.7 mg of purified protein which was identified as CBG by partial amino acid sequencing. SDS-PAGE with reduction and sucrose gradient sedimentation under non-denaturing conditions gave M_r of 58,000 and 46,000, respectively, as expected of rat CBG. This protein bound tritium labeled cortisol, progesterone, dihydrotestosterone (DHT) or 17β-estradiol (E_2) with K_a of 1.09×10^9, 1.28×10^8, 1.41×10^5 and 1.11×10^5 M^{-1}, respectively. The order of steroid binding plus the magnitude of the K_a was consistent with other reports of rat CBG. Unexpectedly, this same protocol yielded 6 mg of another protein. It bound labeled DHT, E_2, progesterone or cortisol with K_a of 4.14×10^7, 2.42×10^7, 8.33×10^4 and 5.55×10^4 M^{-1} respectively. This order of steroid binding was the same as human SHBG but the K_a were substantially lower. The K_a of the sex hormone binding to the rat protein were nearly identical to those seen with horse SHBG Type II. SDS-PAGE after reduction yielded three forms of M_r 60,000, 54,000 and 29,000. When analyzed by sucrose gradient sedimentation under non-denaturing conditions, all three forms sedimented as a single M_r 110,000 component. A brief treatment with a bifunctional chemical cross linking agent converted all three forms to reduction resistant $M_r \geq 110{,}000$ products. Attempted sequencing indicated the protein was N^{α} blocked. Enzymatic fragmentation followed by reverse phase HPLC yielded five peptides which were sequenced and found to share ≤ 40% amino acid homology with human or rabbit SHBG or with rat testicular androgen binding protein. Despite its apparent low homology to SHBG, antibodies against human SHBG cross reacted with the rat protein. Western analysis and tritium labeled DHT binding studies demonstrated this protein in the serum from adult rats of both sexes. Based on the results presented, we have propose that the serum from adult rats of both sexes contains a significant concentration of a SHBG-like protein which has not been characterized previously. We have tentatively named this new protein SHBG Type II. By all criteria applied, rat SHBG Type II was highly similar to horse SHBG Type II.

We are now in the process of purifying SHBG Type II from blood bank (HIV tested) human plasma collected from adult males. As will be described below, our identification of the presence of Type II SHBG in human plasma came from Western immunoblotting with antibody raised against this form horse SHBG Type II. We also plan to purify human SHBG Type I by the standard testosterone affinity chromatography now in use in other laboratories. This is critically necessary for direct comparisons of SHBG Type I and SHBG Type II in cell growth assays and receptor binding studies.

As yet, we do not know if SHBG Type I and SHBG Type II share functional properties with regard to control of breast cancer cell growth. The purification of both forms is a major goal of the next year. This effort will be facilitated by our new FPLC. We anticipate this will require one year. This additional work will delay the completion of TASK 1.

2. Task 1(d): ***Rabbit polyclonal antisera will be raised against ovalbumin conjugates of EMSF peptides synthesized by solid phase methods.***

Because of the relative abundance of the proteins isolated (*i.e. milligrams per preparation)*, we did not need to raise antiserum using synthetic peptide/conjugation methods. Instead, we used purified horse SHBG Type II. To obtain a highly specific antiserum, we first did preparative SDS-PAGE to separate the three subunits. Approximately 3 mg of the M_r 58,000 subunit were isolated and used to raise antisera in two rabbits (done under contract by HTI Bio-Products, Ramona, CA). With this antiserum, we have initiated a number of very important studies. These are summarized next:

❑ We have demonstrated by Western analysis that this antiserum recognized all three subunits of purified horse SHBG Type II. This observation indicated that all three subunits were either related structurally or most likely were processed derivatives of one main form. This information had considerable value with respect to our cloning effort. It suggested that only one cDNA coded for all three subunits of the complete M_r 108,000 complex.

❑ Using Western methods, we demonstrated that the antiserum against horse SHBG Type II identified the same size proteins and/or subunits in adult equine, porcine, bovine, rat, human and ovine serum. However, fetal bovine and fetal horse serum did not show any cross reacting materials. Apparently, SHBG Type II is common in the adults of many species but not in fetal plasma.

❑ Western procedures demonstrated that the antiserum against horse SHBG Type II also recognized all three subunits of purified rat SHBG Type II. This meant that cDNA cloning by immunological methods with rat (liver) expression libraries was possible with this antiserum.

❑ The same Western immunoblotting also confirmed that human serum contains SHBG Type II in amounts which may match those of horse or rat serum (*i.e.* 4 to 5 mg/liter). The cross reaction was particularly pronounced and specific with serum from human males. These analyses suggest that males may have relatively higher levels of Type II than females. Indeed, the level of Type II may be lowest in pregnant female serum, which is most often used to isolate SHBG Type I. The observation of cross reactivity between the antiserum against horse SHBG Type II and human SHBG Type II means that molecular cloning from human (liver) expression libraries is entirely possible!

❑ The antiserum is suitable to development of a highly specific radioimmunoassay (RIA) for SHBG Type II in plasma. This will facilitate our endocrine physiology studies to be described below.

3. Task 1(e): ***Immunoadsorption will be used to confirm the relationship between amino acid sequence and activity.***

We are now establishing the conditions needed to ask if the immunological removal of SHBG Type II and CBG from serum causes a loss of estrogen and progesterone regulated human breast cancer cell growth in culture. An antiserum against CBG is now being raised by HTI Bio-Products, Ramona, CA. The CBG used as antigen was further purified by sucrose gradient sedimentation to separate possible trace levels of SHBG. By SDS-PAGE analysis at high sample loads, the CBG antigen was pure. This antiserum, and the antiserum against SHBG Type II described above, will be used to prepared chemically conjugated Sepharose-IgG columns. Samples of serum will be passed through the columns and the effluent assayed for removal of the specific protein by measuring tritium labeled steroid binding, effect on cell growth plus and minus estrogens and by RIA.

In addition, we are determining the conditions for immunoabsorption of SHBG Type II from serum-free defined culture medium to ask if its removal causes the loss of the serum protein-induced inhibition of growth as well as the loss of estrogen stimulation. For these studies we have already found the conditions to saturate Protein A-Sepharose with the rabbit antiserum against horse SHBG Type II. We are now titrating the amount of this Sepharose needed to remove specific amounts of ^{125}I-SHBG. When this control is completed, we will begin the in culture growth tests of the effects of Sepharose with pre-immune serum versus the gel with antiserum. These studies are progressing as planned.

4. Tasks 1(f) and 1(g): ***(f) The tissue of origin of EMSF will be sought in mouse organ extracts by Northern blotting for m-RNA. (g) The effects of estrogen status on EMSF concentration in mouse blood will be assessed by ELISA or RIA.***

We have elected to change the animal species used in our future studies. Approval for the use of Sprague-Dawley (SD) outbred rats has been obtained from the University of Texas Health Science Center Animal Welfare Committee (protocol approval number HSC-AWC-95-141). We originally planned this study with mice. Because of our discovery of a new form of SHBG in rat, and our identification of a similar form in human plasma, we wished to change our animal model. Also, rats yield more plasma per animal which has already greatly facilitated the next studies.

We have collected the internal organs and plasma from SD rats of a number of ages both sexes. Six to eight rats of each of the following age groups were used: ages 20-21 days, 36-40 days, 55-60 days, 85-128 days and retire breeders of ≥270 days. The organs were removed, rinsed briefly with saline and frozen immediately on dry ice. The plasma from each animal was kept separate. All samples are now stored at -80°C.

We have not yet begun the Northern analysis of ploy (A^+) to determine the tissue(s) of origin of SHBG Type II. However, since SHBG Type I is known to be produced nearly exclusively by liver, it seems entirely likely that this will be the case with SHBG Type II. The demonstration of this fact awaits a cDNA probe for SHBG Type II.

Using a filter membrane (DE 81) binding assay method which measures ^{3}H-DHT bound to SHBG, the concentrations of this protein in the plasma were estimated. We are now developing a RIA using ^{125}I-SHBG and the antiserum against horse SHBG Type II. In addition, we plan in the near future to use highly purified rat SHBG Type II to obtain another rabbit antiserum for RIA and other immunological studies.

During the course of pursuing Task 1(g), we uncovered a remarkable correlation between mammary carcinogenesis and SHBG Type II. The serum levels of SHBG Type II in rats were 5 to7 μg/ml at day 20-21 (weaning on day 19). This concentration fell sharply to 1 to 2 μg/ml during puberty at between 30 to 65 days of age. The levels rose again in adults of 128 days and reached a maximum of 5 to 8 μg/ml in retired breeders. This pattern was true with both males and females implying that puberty related sex hormones (or pituitary hormones) regulated the plasma levels of SHBG Type II. Also, we have confirmed these same changes using Western immunoblotting.

The puberty related depression in concentration correlated well with the known time "window" in which mammary carcinogens are most effective. Numerous studies with different carcinogens have established that young female rats of ages 40 to 70 days are most susceptible to tumorigenesis. This susceptibility correlated strongly with a marked increase in mammary DNA synthesis during puberty (most likely due to the increased secretion of estrogens). DNA synthesis is essential for mammary carcinogenesis. Our observations raise the possibility that SHBG Type II is a natural inhibitor of normal breast epithelial cell growth. When SHBG Type II levels are low, DNA synthesis may increase and hence increase the susceptibility of mammary cells to carcinogens. We plan to expand these unexpected but very important observations.

5. Tasks 1(h) and 1(i): ***(h) cDNA libraries will be screened for EMSF containing plasmids. (i) If the complete sequence of EMSF is not known, we will determine it by dsDNA dideoxy chain termination sequencing.***

For the following reasons, we propose that the form of SHBG isolated by our laboratory is a new molecule which must be characterized my cDNA cloning and sequencing:

❑ Our partial amino acid sequencing of horse and rat SHBG Type II showed them to be at best 35% homologous to SHBG Type I or to rat androgen binding protein.

❑ Both horse and rat SHBG Type II are N^{α} blocked. Human and rabbit SHBG Type I are not.

❑ SHBG Type II has a higher molecular weight than Type I and a different subunit structure.

❑ SHBG Type II binds steroid hormones with a 10 to 20 fold lower affinity than Type I. Because of this low affinity, it is unlikely that Type II acts as a physiological carrier of sex steroids in plasma. It must serve other function(s).

To carry out cDNA molecular cloning, we will use one approach with two libraries. First, we have obtained fresh frozen liver from adult male and female horses. The tissue was from a horse abattoir and flash frozen within minutes after removal of the liver from the carcass. The tissue has is now being used to prepare poly (A^+) RNA for a new λZAP cDNA library by the methods described (69). In addition, we purchased a λZAP cDNA library made from male rat liver poly (A^+) RNA (Stratagene). We have started expression screening of the rat liver library using the anitserum against horse SHBG Type II. We have already encounter one problem. Even though our antiserum is highly specific when measured against complete serum, it still recognizes four *E. coli* proteins. We have two options to correct this problem. First, we will affinity purify our antiserum using SHBG Type II covalently cross linked to cyanogen bromide activated Sepharose. If this solves the problem, we will proceed as planned in the original application. If it does not, we will preabsorb the antiserum with *E. coli* extracts. We feel one of these approaches will be effective.

It is important to note that we have increased the scope of Tasks 1(h) and 1(i) significantly since the original application. We now plan to obtain the complete cDNA sequences of rat, human, horse and mouse SHBG Type II. Aside from providing a strong comparative molecular basis for the identification of a new form of SHBG, the sequences from the different species will be used to achieve specific goals. The human cDNA sequence can be used to obtain a genomic clone for studies of mutations, natural variations and chromosomal localization. Rat cDNA will be used to characterize the tissue of origin by the sensitive and accurate S1 nuclease protection assay as well as to complete our proposed study of the endocrine physiology of SHBG Type II in this animal model. The horse clone will give us the complete structure of the most readily available source of SHBG Type II. We plan to conduct many of the receptor and cell culture experiments with this relatively abundant serum protein. Knowledge of its complete structure will be very useful. Finally, in the future, we plan to conduct mouse knockout experiments. This will provide vital information concerning the *in vivo* function of Type II in normal mammary gland development as well as identify a possible role in tumor incidence and growth.

6. Tasks 2(a), 2(b), 2(c) and 2(d): ***(a) ^{125}I-EMSF will be made by the chloramine T reaction. (b) Specific ^{125}I-EMSF binding will be investigated with estrogen sensitive breast cancer cells. The experimental parameters will be time, concentration, temperature, and effects of estrogens in the culture medium. Scatchard analysis is expected to give K_D values and numbers of sites per cell. (c) Chemical cross-linking with ^{125}I-EMSF followed by SDS-PAGE and autoradiography will be used to identify the M_r of the specific ligand binding receptor. (d) Internalization will be studied to ask if EMSF undergoes receptor mediated endocytosis.***

We have identified SHBG receptors on MTW9/PL2 rat mammary tumor cells and T47D human breast cancer cells in culture. Radio iodinated SHBG was prepared by a modification of the chloramine T method used by us before. There was no problem preparing very high specific activity SHBG which retained biological potency. We have established the temperature, time and concentration dependence of ^{125}I-SHBG binding. For these studies specific binding of radio labeled SHBG was measured as total binding minus binding in cultures containing a 200-fold excess of unlabeled SHBG. Binding at 37°C reached a maximum at 2 hours. At 23°C, a maximum was

reached at 3 hours. Incubations at 4°C showed a maximum at 6 to 10 hours. At the two higher temperatures, maximum binding was followed by a rapid decline suggesting receptor mediated endocytosis. This was blocked at 4°C. When cells were incubated at 37°C in the presence of iodinated SHBG, low molecular products appeared in the culture medium which were small peptides containing the radio labeled iodine. This observation supported the view that SHBG Type II undergoes receptor mediated endocytosis.

Scatchard analysis indicated a K_d of 1.0 nM for SHBG binding with ~30,000 sites per cell. All of the studies described were done with ^{125}I-SHBG from which steroid hormone had been removed by charcoal treatment at 34°C. When labeled SHBG was saturated with DHT or estradiol before initiating the binding studies at 4°C, all binding to receptors was blocked. When saturating concentrations of dihydrotestosterone or estradiol were added to the incubation conditions at 37°C, specific binding also was blocked. The data obtained with MTW9/PL2 cells confirm that only steroid hormone depleted SHBG binds to receptors. When sex steroid hormone is bound to free SHBG, the ligand no longer associates with receptor.

Chemical cross linking of ^{125}I-SHBG to its receptors is in progress. We have experience some difficulty with this study. The problem may be related to the type of bifunctional reagent chosen for the cross linking (*i.e.* BS^3). More work with different bifunctional reagents is planned in the very near future.

The presence of CBG receptors on MTW9/PL2 rat mammary tumor cells was established by the same methods outlined above for SHBG. These studies confirmed approximate 100,000 receptor sites per cell. Additional work is in progress to define other binding parameters of ^{125}I-CBG and to further explore the molecular characteristics of the receptors by chemical cross linking with appropriate bifunctional reagents.

7. Additional Work Accomplished:

we extended our study of the regulatory effects of SHBG Type II to completely serum-free conditions. The purpose of this approach was to identify Type II effects in the complete absence of other potential co-inhibitors in serum. In a previous study, we had reported the development of a serum-free hormonally defined medium for MTW9/PL2 cells. This medium contained F12-DME supplemented with 0.5 mg/ml bovine serum albumin, 10 μM ethanolamine, 10 ng/ml selenous acid, 50 μg/ml apotransferrin, 10 μg/ml diferric transferrin, 10 μg/ml insulin and 1.0 nM T_3. Growth in this medium was logarithmic for 10 days with a cell population doubling time of ~ 30 hours. The addition of 10 nM 17β-estradiol (E_2) increased the growth rate by 10 to 15%. When SHBG was added, 1.5 to 2.0 μg/ml completely inhibited growth. This response was reversed in part by 1.0×10^{-13} M E_2. Addition of 1.0×10^{10} M E_2 promoted optimum growth equal to the estrogen response seen in serum containing cultures. A series of investigations were done to show that horse SHBG Type II did not associate with E_2 in culture medium under concentrations reached 500 nM. The experiments presented indeed have confirmed that SHBG Type II is a potent inhibitor of estrogen responsive mammary tumor cell growth and that estrogens reverse the inhibition.

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8. Manuscripts Submitted or in Preparation:

(1) Moreno-Cuevas JE & Sirbasku DA. Steroid hormone-responsive growth of the MTW9/PL2 rat mammary tumor cells in culture medium supplemented with charcoal-dextran extracted serum. (submitted to *Molecular and Cellular Endocrinology*)

(2) Moreno-Cuevas, JE & Sirbasku DA. Estrogen-dependent growth of the MTW9/PL2 rat mammary tumor cells in culture. Evidence for a role of a calcium related inhibitory serum protein. (submitted to the *Journal of Steroid Hormone Biochemistry and Molecular Biology Molecular*).

(3) Sirbasku, DA, Tanji, M & Moreno-Cuevas, JE. Purification and characterization of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) from equine serum. (To be submitted to *Biochemistry*).

(4) Sirbasku, DA, Tanji, M & Moreno-Cuevas, JE. Properties of sex hormone-binding globulin (SHBG) and corticosteroid-binding globulin (CBG) purified from adult rat serum by cortisol-agarose and phenyl-Sepharose chromatography. (To be submitted to *Endocrine*).

(5) Moreno-Cuevas JE & Sirbasku DA. Identification of sex hormone-binding globulin (SHBG) receptors on MTW9/PL2 rat mammary tumor cells. (submitted to *Biochemistry and Biophysics Research Communications*).

(6) Moreno-Cuevas JE & Sirbasku DA. Sex hormone-binding globulin (SHBG) regulation of estrogen-responsive MTW9/PL2 rat mammary tumor cell growth in serum-free hormonally defined medium. (To be submitted to the *Proceeding of the National Academy of Science, USA*).

(7) CONCLUSIONS

A. Discoveries from this Project

OUR GROUP HAS MADE TWO MAJOR DISCOVERIES IN THE FIRST 24 MONTHS OF THIS PROJECT. THEY ARE:

(1) WE ARE THE FIRST TO IDENTIFY A NEW FORM OF SHBG (TYPE II) WHICH HAS A DIRECT ROLE FOR IN THE REGULATION OF STEROID HORMONE DEPENDENT BREAST CANCER CELL GROWTH.

(2) WE HAVE DEMONSTRATED IN CULTURE THAT CBG IS ALSO A NEGATIVE REGULATOR. THIS RAISES VERY INTERESTING POSSIBILITIES WITH REGARD TO STRESS AND BREAST CANCER.

THE RESULTS RELATED TO SHBG TYPE II HAVE BEEN VERBALLY COMMUNICATED TO COLONEL IRENE M. RICH, DNSc., DIRECTOR, DEPARTMENT OF DEFENSE, BREAST CANCER RESEARCH PROGRAM, DEFENSE WOMEN'S HEALTH RESEARCH PROGRAM.

A. MAJOR CONCLUSIONS AND IMPLICATIONS

(1) SHBG Type II is a physiologically important negative regulator of estrogen responsive breast cancer cell growth. This discovery is important because it is the first to identify a specific serum-borne entity which demonstrates such a function.

(2) CBG is another major physiologically important negative regulator of progestin and estrogen responsive breast cancer cell growth. This plasma glycoprotein has not previously been recognized as having a function in breast cell growth regulation.

(3) Our laboratory has identified a new area of breast cancer research. We have established that breast cell growth can be blocked by physiologically available serum-borne inhibitors and that steroid hormone responsive cell types remain quiescent until stimulated by estrogens or progestins.

(4) Our data raise the possibility that the lack of this new form of SHBG in females may be a major predisposing factor to breast cancer.

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B. MAJOR CHANGES IN TASKS 2(g) and 2(h): *(g) The Mabs will be used to screen cDNA libraries from estrogen sensitive cells to obtain plasmids for dideoxy dsDNA sequencing of the complete receptor. (h) Transfection with full length receptor cDNA will be evaluated to determine if estrogen insensitive cells can be reverted to negative control by EMSF.*

Because the work of **Task 1** and **Task 2** has nearly doubled with the discovery of a new form of SHBG as a steroid hormone reversible inhibitor of breast cancer cell growth, and the discovery that CBG also serves this same function, we have had to reassess how much additional work can be accomplished in the next 24 months. This is especially pertinent to budget issues.

Since originally proposing it, we now recognize that Task 2(g) is a very major and expensive undertaking which must be treated as a separate project. Also, the monoclonal antibody approach to the isolation of receptors is no longer the most effective means of accomplishing this goal. To isolate the cDNA of the SHBG Type II receptor, there are now much better methods. With proper expression libraries from SHBG Type II receptor positive cells, it is now more efficient to use expression cloning with receptor negative mammalian cells in culture. This approach is highly feasible. It is also labor intensive and expensive. For these reasons, we have submitted a new IDEA application to the US Army Breast Cancer Program (it was reviewed in September, 1996). In it we projected the following studies:

❑ We proposed to isolate the SHBG Type II receptor cDNA by expression cloning with mammalian cells in culture. The methods chosen were those which already have been applied to more that twenty receptors.

❑ The receptor cDNA will be used to obtain a genomic clone of the receptor. This will be used to screen tumor samples for mutations and alterations related to hormone responsive and autonomous breast cancers.

❑ We plan to localize the gene for the SHBG receptor to a specific chromosome.

These studies were not part of the original plan in this US Army Breast Cancer Program GRANT NUMBER: DAMD17-94-J-4473.

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pp 72-74 -- End labeling of synthetic probes.
pp 75-78 -- Hybridization with synthetic ^{32}P end-labeled probe.
pp 130-135 -- Guanidine isothiocyanate preparation of total RNA.
pp 143-146 -- Formaldehyde gel for electrophoretic separation of RNA and Northern blot
pp 199-207 -- Generation of cDNA insert from eukaryotic mRNA.
pp 211-215 -- Plating and screening of λgt10 and λgt11 packaged inserts.
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